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INDUCTION OF APOPTOSIS BY THE PRO-APOPTOTIC BCL-2 FAMILY MEMBER BOK IS NOT ANTAGONIZED BY EITHER BCL-2 OR BCL-XL, SUGGESTING THAT BOK MIGHT HAVE A UNIQUE ROLE IN THE APOPTOTIC CASCADE. WE SHOWED HERE THAT HUMAN BOK IS THE ONLY MEMBER OF THE BCL-2 FAMILY TO HAVE A LEUCINE-RICH SEQUENCE INDICATIVE OF A NUCLEAR EXPORT SEQUENCE WITHIN ITS BH3 DOMAIN. BOK WAS DETECTED IN BOTH THE NUCLEUS AND THE CYTOPLASM OF HEK 293T CELLS, HELA CELLS, AND BREAST CANCER CELLS, AND ITS NUCLEAR CONCENTRATION INCREASED AFTER TREATMENT OF THOSE CELLS WITH LEPTOMYCIN B, AN INHIBITOR OF THE EXPORTIN CRM1. MUTATING THE NUCLEAR EXPORT SEQUENCE OF BOK RESULTED IN AN INCREASE IN ITS NUCLEAR LOCALIZATION AND APOPTOTIC ACTIVITY. WE ALSO FOUND THAT CRM1 INTERACTED WITH WILD-TYPE BOK BUT NOT WITH THE MUTATED FORM. THESE RESULTS SUGGEST THAT NUCLEAR EXPORT OF BOK IS A REGULATED PROCESS MEDIATED BY CRM1, AND CONSTITUTES THE FIRST REPORT OF A LINK BETWEEN THE APOPTOTIC ACTIVITY AND NUCLEAR LOCALIZATION OF A PRO-APOPTOTIC MEMBER OF THE BCL-2 FAMILY.

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Introduction

Central to apoptosis is the activation of caspases [1,2]. One apoptotic pathway that activates caspases is the intrinsic apoptotic pathway, which induces changes in mitochondrial membrane permeability and the release of mitochondrial proteins such as cytochrome C [3], which binds to the adaptor protein Apaf-1 and in the presence of dATP activates pro-caspase 9. Activated caspase 9 activates caspase 3, leading to apoptosis [4].

The Bcl-2 family of proteins are key regulators of the intrinsic apoptotic pathway. Members of the Bcl-2 family play a pivotal role in regulating apoptosis by controlling the mitochondrial changes associated with the release of cytochrome C [5]. Bcl-2, Bcl-x_L, Bcl-w, and Mcl-1 promote cell survival by inhibiting the release of cytochrome C while Bax, Bak [the Bcl-2 Homology [BH] domains 1–3 group] or Bad, Bid, Bik, Bim, Puma, Noxa, and others [the BH3 domain-only group] induce cell death by promoting the release of cytochrome C. The BH3 domain-only group serves as sensors of apoptotic signals [5-7]. Predominantly cytosolic in healthy cells, Bax translocates to the mitochondria in response to an apoptotic stimulus, where it promotes cell death by altering the permeability of the mitochondrial membrane [8,9]. Bax and Bak demonstrate functional redundancy at the mitochondria in that one (but not both) must be functional for apoptosis-related mitochondrial changes to occur [10,11]. Recently, associations were noted between the apoptotic functions of Bax and Bak and the release of calcium from the endoplasmic reticulum [10,12]. Although Bcl-2 and Bcl-x_L have been detected in the membranes of the endoplasmic reticulum, mitochondria, and nucleus [13-15], their major function is at the mitochondrial membrane, where they oppose the mitochondrial changes initiated by Bax or Bak. There is at present no evidence linking nuclear localization of any member of the Bcl-2 family with their anti or pro-apoptotic function. Rat Bok a member of the Bcl-2 family, is similar to Bax

and Bak in that it includes BH1, BH2, and BH3 domains and a COOH-terminal transmembrane region [16,17]. Like some pro-apoptotic Bcl-2 family members, the expression of Bok seems to depend on p53 [18]. However, Bok fails to form heterodimers with Bcl-2 or Bcl-x_L and its apoptotic activity is not antagonized by Bcl-2 [16], suggesting that Bok might have a unique role in the apoptotic cascade. We studied this possibility by using the human homolog of rat Bok (termed hBok). Comparison of the gene bank–deposited cDNA sequences of rBok and hBok showed that these two genes differ by only nine amino acid residues. We show that this difference does not affect the apoptotic activity of either protein. The BH3 domain of hBok contains the highly conserved amino acid residues present in all anti- and pro-apoptotic members of the BCL-2 family [19-21]. However, unlike other members of this family, hBok has in its BH3 domain a short leucine-rich stretch of amino acids representative of a nuclear export signal (NES) [22].

BODY:

A. Specific Aims: (NO CHANGES)

Specific Aim 1: To confirm hBok-mediated transformation suppression in breast cancer cells

Specific Aim 2: To investigate the mechanisms of hBok-induced apoptosis

Specific Aim 3: Todevelop a tumor specific promoter using hTERT promoter driven hBok to examine the preclinical effect of hBok for breast cancer gene therapy.

B. STUDIES AND RESULTS

Specific Aim 1. To confirm hBok-mediated transformation suppression in breast cancer cells.

Rat Bok had been shown to an effective apoptotic molecule. Knowing the complication of using a murine gene for gene therapy, we cloned the human homolog of rat Bok and observed that both genes were 95% identical. To investigate if this 5% difference affects the apoptotic activity of hBok we expressed both proteins in the human embryonic kidney (HEK) 293T cells and observed by the use of multiple apoptotic assays, that both hBok and rBok have apoptotic activity, with hBok being the stronger of the two. These results suggest that the minor differences in the amino acid residues within the hBok protein have no negative influence on its apoptotic activity. We next tested the ability of hBok to inhibit the growth of breast cancer cell lines in an in-vitro tissue culture system and observed that hBok inhibited the growth of these breast cancer cells.

Specifc Aim 2: To investigate the mechanism of hBok-induced apoptosis

hBok is structurally very similar to Bax and is also a member of the Bax group of pro-apoptotic Bcl-2 molecules. While investigating if endogenous hBok, like Bax, is located in the cytoplasm of cells under normal growth conditions, we unexpectedly found hBok to be predominantly located in the nucleus of MDA-MB-231 breast cancer cells but equally distributed between the cytoplasm and nucleus of HeLa cells As expected, Bax was present only in the cytoplasm in both cell lines. Similar observations were made by immunocytochemical analysis of Flag-tagged hBok-transfected HeLa and MDA-MB-231 cells. We also screened breast, ovarian, and pancreatic cancer cells and again noted significant percentages of cells demonstrating nuclear localization of hBok under normal growth conditions. We conclude that hBok is a nuclear protein in the cells we examined. Unlike other members of the BAX family the BH3 domain of Bok contains a nuclear export signal(NES). Since the export of nuclear proteins that contain this

leucine-rich NESs is mediated by the transporter protein CRM1 we asked if the if the nuclear export of hBok is mediated by Crm1. As shown by work presented in our manuscript we clearly show an association between hBok and CRM1. As further confirmation of this interaction, we used the knowledge that CRM1-mediated export of proteins can be inhibited by leptomycin B, an unsaturated, branched-chain fatty acid that specifically inhibits CRM1 and showed that hBok accumulated in the nucleus of leptomycin B–treated cells to a greater extent than in untreated cells. These results suggest that the transport of hBok across the nuclear membrane is a Crm1–mediated process. To extend these observations, we generated an NES mutant that we expected would be unable to interact with Crm1 by substituting the presumably critical leucine residues within the putative NES sequence (^{72}Leu and ^{74}Leu) with alanine. We observed that mutating the NES of hBok resulted in significant sequestration of this protein in the nucleus supporting our conclusion that the translocation of hBok from the nucleus to the cytoplasm is mediated by Crm1. We next wanted to determine if the observed nuclear–cytoplasmic translocation of hBok was relevant to its apoptotic activity. Our tissue culture system using HEK 293T cells transfected with a pcDNA3 vector or vector-expressing either wild-type or mutant hBok showed tha mutant hBok had greater cell-killing activity than the wild-type hBok. To extend these findings, we studied the dynamics of cell death in wild-type– and mutant-hBok–transfected cells over a 24-hour period. Using the trypan blue exclusion assay we once again showed mutant hBok had a stronger effect on killing the cells compared to wildtype. Based on FACS Analysis we attribute the higher potency of mutant hBok to its ability to induce apoptosis more quickly than the wild type. This result implies that accumulation of hBok in the nucleus enhances its killing potential. Similar results were obtained with the breast cancer cell lines MDA-MB-435 (MDA-MB-231 and chinese hamster ovary cells.

Specific Aim 3: To develop a tumor specific promoter using hTERT promoter hBok to examine the preclinical effect of hBok for Breast cancer gene therapy.

We were unable to accomplish this Aim due to time restraints as most of our time was spent generating antibodies to hBok that were ineffective, as well as expanding our study on the possible mechanism of action of hBok. In addition, our initial attempts at trying to drive the expression of hBok by a tumor specific promoter were not successful.

KEY RESEARCH ACCOMPLISHMENTS

- The pro-apoptotic Bcl-2 member hBok inhibits the growth of breast cancer cells
- Unlike other members of this family hBok is present in both the cytoplasm and nucleus of cells growing under normal conditions.
- The nuclear cytoplasmic translocation of hBok is mediated by CRM1
- Sequestration of hBok in the nucleus enhances its apoptotic activity.

REPORTABLE OUTCOMES

Our studies on the nuclear cytoplasmic transport of hBok and the association of its intracellular localization to its activity has currently been submitted to Oncogene. The Title of this manuscript is "Nuclear translocation of the pro-apoptotic Bcl-2 family member Bok induces apoptosis" A copy of this manuscript is enclosed.

CONCLUSION:

In this report we show that endogenous hBok is present both in the cytoplasm and nucleus of cancer cells. In addition we show that the nuclear cytoplasmic translocation of of hBok is not by simple diffusion but is an active process mediated by Crm1. Our results also suggest that the accumulation of hBok in the nucleus of cells may enhance its apoptotic activity. The Bcl-2 family of anti- and pro-apoptotic molecules has critical roles in regulating apoptosis. In a recent review [23], the authors proposed that the anti-apoptotic members could function as oncogenes and the pro-apoptotic members as tumor suppressors. It has been well documented in transgenic mouse models that overexpression of Bcl-2 contributes to the onset of B-lymphoid tumors [24 25], breast tumors [26], and pancreatic cell tumors [27]. Overexpression of Bcl-2 also renders tumor cells refractory to chemotherapy and radiation [28-30]. In an attempt to identify a tumor suppressor that could be successfully used against a broad variety of tumors, including those that express high levels of Bcl-2, we selected the little-studied pro-apoptotic Bcl-2 family member Bok. This choice is of clinical significance given the over expression of Bcl-2, an inhibitor of apoptosis, by many types of human tumors [31-34] and the lack of antagonism of Bok activity by Bcl-2 [16]. Although the mechanism regulating the cytoplasmic–nuclear transport of hBok remains elusive and needs further study we propose that a good understanding of the mechanism of Bok-induced apoptosis together with successful targeting of the NES mutant of hBok to breast cancer might be beneficial to the treatment of this disease.

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Nuclear translocation of the pro-apoptotic Bcl-2 family member Bok induces apoptosis

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Running title: Nuclear Bok has apoptotic activity

Key words: apoptosis, nuclear transport, pro-apoptotic Bcl2 family, nuclear export signal

ABSTRACT

Induction of apoptosis by the pro-apoptotic Bcl-2 family member Bok is not antagonized by either Bcl-2 or Bcl-XL, suggesting that Bok might have a unique role in the apoptotic cascade. We showed here that human Bok is the only member of the Bcl-2 family to have a leucine-rich sequence indicative of a nuclear export sequence within its BH3 domain. Bok was detected in both the nucleus and the cytoplasm of HEK 293T cells, HeLa cells, and breast cancer cells, and its nuclear concentration increased after treatment of those cells with leptomycin B, an inhibitor of the exportin Crm1. Mutating the nuclear export sequence of Bok resulted in an increase in its nuclear localization and apoptotic activity. We also found that Crm1 interacted with wild-type Bok but not with the mutated form. These results suggest that nuclear export of Bok is a regulated process mediated by Crm1, and constitutes the first report of a link between the apoptotic activity and nuclear localization of a pro-apoptotic member of the Bcl-2 family.

Introduction

Central to apoptosis is the activation of caspases (Strasser *et al.*, 2000) (Green, 2000). One apoptotic pathway that activates caspases is the intrinsic apoptotic pathway, which induces changes in mitochondrial membrane permeability and the release of mitochondrial proteins such as cytochrome C (Green and Reed, 1998), which binds to the adaptor protein Apaf-1 and in the presence of dATP activates pro-caspase 9. Activated caspase 9 activates caspase 3, leading to apoptosis (Desagher *et al.*, 1999).

The Bcl-2 family of proteins are key regulators of the intrinsic apoptotic pathway. Members either promote cell survival (Bcl-2, Bcl-x_L, Bcl-w, Mcl-1) or cell death [(Bax, Bak, the Bcl-2 Homology [BH] domains 1–3 group), or (Bad, Bid, Bik, Bim, Puma, Noxa, and others the BH3 domain-only group)]. The BH3 domain-only group serves as sensors of apoptotic signals (Huang and Strasser, 2000) (Gross *et al.*, 1999) (Shimizu *et al.*, 1999). Bcl-2, Bcl-x_L, Bax, and Bak contain a stretch of hydrophobic amino acids at their COOH termini that serves to anchor these proteins into organelle membranes (Borner *et al.*, 1994) (Nguyen *et al.*, 1994). Predominantly cytosolic in healthy cells, Bax translocates to the mitochondria in response to an apoptotic stimulus, where it promotes cell death by altering the permeability of the mitochondrial membrane (Goping *et al.*, 1998) (Wolter *et al.*, 1997). Bax and Bak demonstrate functional redundancy at the mitochondria in that one (but not both) must be functional for apoptosis-related mitochondrial changes to occur (Zong *et al.*, 2003) (Lindsten *et al.*, 2000). Recently, associations were noted between the apoptotic functions of Bax and Bak and the release of calcium from the endoplasmic reticulum (Scorrano *et al.*, 2003) (Zong *et al.*, 2003). Although Bcl-2 and Bcl-x_L have been detected in the membranes of the endoplasmic reticulum, mitochondria, and nucleus (Borner *et al.*, 1994) (Nguyen *et al.*, 1994) (Kaufmann *et al.*, 2003),

their major function is at the mitochondrial membrane, where they oppose the mitochondrial changes initiated by Bax or Bak.

Rat Bok, also known as mtd, a little studied pro-apoptotic member of the Bcl-2 family, is similar to Bax and Bak in that it includes BH1, BH2, and BH3 domains and a COOH-terminal transmembrane region (Hsu *et al.*, 1997) (Inohara *et al.*, 1998). Like some pro-apoptotic Bcl-2 family members, the expression of Bok seems to depend on p53 (Yakovlev *et al.*, 2004). However, Bok fails to form heterodimers with Bcl-2 or Bcl-x_L and its apoptotic activity is not antagonized by Bcl-2 (Hsu *et al.*, 1997), suggesting that Bok might have a unique role in the apoptotic cascade. We studied this possibility by using the human homolog of rat Bok (termed hBok). Comparison of the gene bank–deposited cDNA sequences of rBok and hBok showed that these two genes differ by only nine amino acid residues. We show here that this difference does not affect the apoptotic activity of either protein.

The BH3 domain of hBok contains the highly conserved amino acid residues present in all anti- and pro-apoptotic members of the BCL-2 family (Adachi and Imai, 2002) (Nakano and Vousden, 2001) (Han *et al.*, 2001). However, unlike other members of this family, hBok has in its BH3 domain a short leucine-rich stretch of amino acids representative of a nuclear export signal (NES) (Gorlich and Kutay, 1999). A consensus for the NES has been defined (Bogerd *et al.*, 1996), but it is now becoming clear that NESs are quite divergent (Heger *et al.*, 2001). Expanding on this observation, we detected hBok in the nucleus of cells and successfully increased its nuclear concentration with leptomycin B, an inhibitor of Crm1. Crm1 mediates the export of leucine-rich, NES-containing proteins (Fomerod *et al.*, 1997). Mutating the NES of hBok resulted in increasing the nuclear concentration of the protein and increasing the potency of its pro-apoptotic effect. This increased apoptotic property of the mutant hBok led to greater

inhibition of cancer cell survival than could be achieved with the wild-type protein. These results suggest that hBok might function at the level of the nucleus, making it a novel member of the pro-apoptotic Bcl-2 family, and suggest a unique mechanism of action associated with this protein.

Results

hBok induces apoptosis

rBok and hBok genes are 95% identical. To investigate if this 5% difference affects the apoptotic activity of hBok, we transiently transfected the pcDNA3 vector, or vector expressing hBok or rBok, into human embryonic kidney (HEK) 293T cells. At 24 h after transfection, cells were harvested and apoptosis determined by terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) (Figure 1a), DNA fragmentation analysis (Figure 1b), Hoechst staining (Figure 1c), and fluorescence-activated cell sorting (FACS) analysis (Figure 1d). The results of these assays clearly demonstrated that both hBok and rBok have apoptotic activity, with hBok being the stronger of the two. These results suggest that the minor differences in the amino acid residues within the hBok protein have no negative influence on its apoptotic activity.

hBok translocates to the nucleus

hBok is structurally very similar to Bax and is also a member of the Bax group of pro-apoptotic Bcl-2 molecules. While investigating if hBok, like Bax, is located in the cytoplasm of cells under normal growth conditions, we unexpectedly found hBok to be predominantly located in the nucleus of MDA-MB-231 breast cancer cells but equally distributed between the cytoplasm

and nucleus of HeLa cells (Figure 2a). As expected, Bax was present only in the cytoplasm. To further confirm the nuclear location of hBok, we used immunocytochemical analysis of Flag-tagged hBok-transfected HeLa and MDA-MB-231 cells (Figure 2b). Our results show quite conclusively that hBok was present predominantly in the nucleus of MDA-MB-231 cells, as demonstrated by the yellow staining of hBok after the merging of the stains for hBok (green) and the nucleus (red). Although hBok colocalized with the endoplasmic reticulum on the periphery of the nucleus in MDA-MB-231 cells (Figure 2c), we still saw hBok in the nucleus, where the green stain predominated. In HeLa cells, in contrast, hBok was equally distributed between the cytoplasm and nucleus. (Figure 2b). Western blot analysis of cytoplasmic and nuclear fractions of Flag-tagged hBok-transfected HEK 293T cells clearly showed both the nuclear and cytoplasmic localization of the protein (Figure 2d). Since endogenous hBok also demonstrated both a cytoplasmic and nuclear distribution in both the MDA-MB-231 breast cancer cells and HeLa (Figure 1a) this ruled out the possibility that the cell type–specific distribution of hBok could be a result of the forced expression of the protein resulting from the transient transfection of the cells and that our observation of the cell type distribution of hBok is a natural phenomenon. We also screened breast, ovarian, and pancreatic cancer cells and again noted significant percentages of cells demonstrating nuclear localization of hBok under normal growth conditions (data not shown). We conclude that hBok is a nuclear protein in the cells we examined.

Nuclear export of hBok is mediated by Crm1

Unique to Bok is the presence of a putative NES, ^{71}LLRLGDELE^{79}, within the BH3 domain of this molecule (Figure 3a). The export of nuclear proteins that contain classical leucine-rich

sequences known as NESs is mediated by the transporter protein CRM1. To determine if the nuclear export of hBok is mediated by Crm1, we immunoprecipitated endogenous Crm1 from HeLa cells and showed by western blotting that Crm1 interacts with hBok (Figure 3b). Similarly, when hBok was immunoprecipitated, Crm1 was detected by western blotting. As further confirmation of this interaction, we used the knowledge that CRM1-mediated export of proteins can be inhibited by leptomycin B, an unsaturated, branched-chain fatty acid that specifically inhibits CRM1. Indirect immunofluorescent staining was carried out on Flag-tagged hBok-transfected MDA-MB-231 cells grown in the presence or absence of leptomycin B (20 ng/ml) (Figure 3c). hBok accumulated in the nucleus of leptomycin B–treated cells to a greater extent than in untreated cells (Figure 3c, left panel). Moreover, a higher percentage of transfected cells treated with leptomycin B showed nuclear hBok than did untreated cells (Figure 3c, right panel), suggesting that the transport of hBok across the nuclear membrane is a Crm1–mediated process.

To extend these observhations, we generated an NES mutant that we expected would be unable to interact with Crm1 and thus would accumulate in the nucleus. Specifically, we used site-directed mutagenesis to generate a mutant in which the presumably critical leucine residues within the putative NES sequence (^{72}Leu and ^{74}Leu) were substituted with alanine (Figure 3d). Indirect immunofluorescent staining of MDA-MB-231 cells transfected with either wild-type hBok or the NES mutant showed that mutating the NES of hBok resulted in significant sequestration of this protein in the nucleus (Figure 3e, left panel). Again, a higher percentage of mutant hBok was found in the nucleus than was wild-type hBok (Figure 3e, right panel). The observation that either mutating the NES sequence of Bok (Figure 3e, left panel) or treating cells transfected with wild-type hBok with leptomycin B (Figure 3c, left panel) led to increased

accumulation of nuclear hBok further confirmed that the nuclear export of hBok is mediated by the exportin Crm1. We conclude that hBok can be detected in the nucleus and that its translocation from the nucleus to the cytoplasm is mediated by Crm1.

Apoptotic activity of nuclear hBok

To determine whether the observed nuclear–cytoplasmic translocation of hBok was associated with apoptotic activity, we transfected HEK 293T cells with a pcDNA3 vector or vector-expressing either wild-type or mutant hBok together with a plasmid expressing luciferase. The concentration of the luciferase-expressing plasmid was set at one-tenth that of the pcDNA3 vectors to improve the probability that cells transfected with the luciferase plasmid were also transfected with the vector. Viability of the transfected cells was determined by analyzing the intensity of luciferase expression at 24 h (Figure 4a). Mutant hBok had greater cell-killing activity than the wild-type hBok. To extend these findings, we studied the dynamics of cell death in wild-type– and mutant-hBok–transfected cells over a 24-hour period; for these studies, cells were harvested at 0 h, 12 h, or 24 h after transfection and their viability assessed by trypan blue exclusion (Figure 4b). Wild-type hBok lowered the cell number to 58% of the control within the first 12 h after treatment; however, during the same 12 h period, mutant Bok reduced the cell number to 41% of the control. We attribute the higher potency of mutant hBok to its ability to induce apoptosis more quickly than the wild type, as shown by the results of a FACS analysis of HEK 293T cells harvested at 24 h after transfection (Figure 4c). This result implies that accumulation of hBok in the nucleus enhances its killing potential. The cell-killing effect of both the NES mutant and wild-type hBok was specific because transfection of the empty vector had minimal effects on cell survival (data not shown). To further demonstrate the difference in

apoptotic activity between wild-type and NES-mutant hBok, we cloned their cDNAs into the pAdTrack vector so that we could independently express the green fluorescence protein (GFP) and the hBok proteins from the same vector, and transfected those plasmids into the breast cancer cell line MDA-MB-435 (Figure 4d). Again, we found that the mutant produced more cell death than the wild-type hBok. Similar results were obtained in the breast cancer cell line MDA-MB-231 and Chinese hamster ovary cells (data not shown). Together, these results indicate that our NES mutant of hBok demonstrated greater apoptotic activity than did the wild-type protein.

Discussion

The Bcl-2 family of anti- and pro-apoptotic molecules has critical roles in regulating apoptosis. In a recent review (Cory and Adams, 2003), the authors proposed that the anti-apoptotic members could function as oncogenes and the pro-apoptotic members as tumor suppressors. It has been well documented in transgenic mouse models that overexpression of Bcl-2 contributes to the onset of B-lymphoid tumors (McDonnell and Korsmeyer, 1991) (Strasser *et al.*, 1993), breast tumors (Jager *et al.*, 1997), and pancreatic cell tumors (Naik *et al.*, 1996). Overexpression of Bcl-2 also renders tumor cells refractory to chemotherapy and radiation (Strasser *et al.*, 1994) (Schmitt *et al.*, 2000) (Sortorius and Krammer, 2002). In an attempt to identify a tumor suppressor that could be successfully used against a broad variety of tumors, including those that express high levels of Bcl-2, we selected the little-studied pro-apoptotic Bcl-2 family member Bok. This choice is of clinical significance given the over expression of Bcl-2, an inhibitor of apoptosis, by many types of human tumors (Liang *et al.*, 1995) (Haldar *et al.*, 1994) (Lkegaki *et al.*, 1994) (Sinicrope *et al.*, 1995) and the lack of antagonism of Bok activity by Bcl-2 (Hsu et al.,

1997).

We showed for the first time a functional relevance to the nuclear localization of a pro-apoptotic member of the Bcl-2 family. Although Bax has been shown to transiently reside in the nucleus, this observation has not been associated with any function (Mandal *et al.*, 1998). Within the BH3 domain of hBok is a short leucine-rich stretch of amino acids corresponding to a putative NES; the most prevalent NESs include a stretch such as this in which the leucine residues are critical for function (Gorlich and Kutay, 1999). A similar leucine-rich sequence is not seen in the BH3 domains of any other member of the Bcl-2 family (Adachi and Imai, 2002) (Nakano and Vousden, 2001]) (Han *et al.*, 2001). Our findings demonstrate that nuclear export of hBok is a regulated process that depends on the nuclear exportin Crm1. This finding led us to investigate whether transport of hBok from the nucleus to the cytoplasm is required for its activation or inactivation. Since the exportin Crm1 mediates the export of leucine-rich NES-containing proteins (Fomerod *et al.*, 1997), we used leptomycin B, an inhibitor of Crm1 function, and showed that inhibition of CRM1 activity resulted in accumulation of nuclear hBok. We then mutated the putative NES of hBok by substituting the essential leucine residues with alanine and showed that not only did this result in nuclear accumulation of Bok, it also enhanced the apoptotic activity of this protein. This finding indicates that the nuclear passage of hBok is most probably not a random event but instead may be required for its apoptotic activity. The observation that nuclear localization is important for hBok apoptotic activity is novel, and this is the first time that a biological function has been associated with the nuclear localization of a member of the Bax family of pro-apoptotic Bcl-2 proteins.

Because Bax is known to translocate from the cytoplasm to the mitochondria upon the induction of apoptosis, we asked if induction of apoptosis would result in translocation of hBok

from the cytoplasm into the nucleus. No such nuclear translocation was observed under our experimental conditions (data not shown). Thus the mechanism regulating the cytoplasmic–nuclear transport of hBok remains elusive and needs further study.

In conclusion, we demonstrated a unique property of a member of the pro-apoptotic Bcl-2 family in showing that its nuclear localization is important for its apoptotic activity. Unlike Bak and Bax, the apoptotic activity of hBok is not antagonized by Bcl-2, and it is tempting to speculate that this lack of antagonism may be due to hBok inducing apoptosis through an unknown pathway independent of the mitochondria and requiring nuclear passage. This putative nuclear function of hBok adds a new link to the already complex mechanisms by which members of the Bcl-2 family regulate apoptosis.

Materials and methods

Cell lines and culture conditions

HEK 293T cells HeLa cells and the human breast cancer cell lines, MDA-MB-435, and MDA-MB-231 were grown in Dulbecco's modified Eagle's medium/F12 medium (GIBCO Laboratories, Grand Island, NY, USA) supplemented with 10% fetal calf serum and penicillin/streptomycin. All cells were incubated in a humidified chamber set at 37°C; the air contained 5% CO_2.

Plasmid construction

To generate the NES mutant of hBok, we constructed specific alanine mutations by using site-directed mutagenesis of a hBok cDNA template that had been cloned into the pcDNA3 mammalian cell expression vector (Invitrogen). The selection primer (5'

GGGACTCTGGGGATCCAAATGACCGACCAAGCG 3') was designed such that the BstB1 site in the pcDNA3 vector was replaced with BamH1. The mutagenic primer designed was 5' GCGCGGTGCTGGCGGCGGCGGGCGATGAGCTG 3', in which the ^{74}LRL^{76} of the putative NES within the BH3 domain was replaced with alanine. Nucleotide sequences were confirmed by DNA sequencing. To express 5' Flag-tagged versions of the wild-type and NES-mutant hBok, fragments of the different hBok cDNAs generated by polymerase chain reaction were subcloned into the pCMV-Tag 2-5 mammalian expression vector (Stratagene). The authenticity of the wild-type and mutant Flag-tagged hBok constructs was confirmed by nucleotide sequencing. The wild-type and NES-mutant hBok cDNAs were then cloned into the AdTrack vector (kindly provided by Dr. Bert Vogelstein of John Hopkins University) by cloning Kpn1/Xba1 Bok fragments into the corresponding sites in the AdTrack vector, and the correct orientation and positioning of the hBok fragments were confirmed by nucleotide sequencing.

Transfection

All DNA transfections were done with a liposome delivery system as previously described (Zou *et al.*, 2002). Cells to be transfected were grown overnight in Dulbecco's modified Eagle's medium/Harris F-12 medium supplemented with 10% fetal calf serum and penicillin/streptomycin. Cells were incubated with the plasmid/liposome complex in serum-free OptiMEM medium (GIBCO Laboratories, Grand Island, NY, USA) for 2 h, after which the OptiMEM was replaced with Dulbecco's modified Eagle's medium/Harris F-12 medium and incubation was continued at 37°C for 24 h or for the times indicated in figure legends.

Immunoflorescence

For immunofluorescence studies, cells were seeded onto four-chamber slides at concentrations of 3–5 x 10^4 cells per chamber and transfected with the control (pCMV-Tag), pCMV-FLAG-hBok, or pCMV-FLAG NES-mutant hBok. At 3, 6, or 9 h, cells were washed three times with ice-cold phosphate-buffered saline (PBS) for 5 min per wash, fixed with 4% paraformaldehyde for 15 min, and permeabilized on ice with 0.2% Triton X-100 for 5 min. The permeabilized cells were then washed in PBS again and treated with 0.1% normal goat serum for 30 min (to minimize the nonspecific adsorption of antibodies) and incubated for 1 h with polyclonal rabbit anti-FLAG (Sigma, St. Louis, MO, USA) and anti-calreticulin (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) antibodies. Cells were then incubated for 1 h with goat anti-rabbit Texas Red secondary antibody (Vector Laboratories, Burlingame, CA, USA). Stained cells were examined by deconvolution microscopy with a Zeiss AxioPlan2 system (Jena, Germany). The green and red fluorescence of fluorescein isothiocyanate (FITC) and Texas Red were visualized and images were captured with a Zeiss AxioPlan2 equipped with a Hamamatsu digital camera.

For leptomycin B treatment conditions, cells were incubated at 37°C for 6 h in Dulbecco's modified Eagle's medium/Harris F-12 medium containing 20 ng/ml leptomycin B. (Sigma, St. Louis, MO, USA)

DNA fragmentation by agarose gel analysis

DNA fragmentation assays were done as described elsewhere (Herrmann *et al.*, 1994).

Nuclear fractionation

For nuclear-cytoplasmic fractionation experiments, HEK 293T cells transfected with the mammalian expression vector pCMV-vector or vector expressing Flag-tagged hBok were harvested 24 h after transfection, and the cytoplasm and nuclear fractions were obtained as described elsewhere (Lin *et al.*, 2001).

TUNEL assay

Apoptotic cells were identified by using the deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay. (Gavrieli *et al.*, 1992). Briefly, HEK 293T cells were seeded at 1 x 10^5 cells per chamber in two-chamber slides for 24 h; transfection was then done with 1 μg DNA delivered by liposomes. Another 24 h later, cells were washed in cold PBS and fixed in 4% paraformaldehyde, and the TUNEL assay was performed. Percentages of apoptotic cells were determined by counting the number of apoptotic cells and dividing by the total number of cells in the field. Data were compiled from a combination of three independent experiments.

FACS analysis

For these experiments, HEK 293T cells were seeded in six-well plates at 1 x 10^5 cells per well for 1 day and then transfected with 2 μg mammalian expression vector pCMV or vector expressing either wild-type of NES mutant delivered by liposomes. After 24 h, adherent and floating cells were harvested by trypsinization, washed twice in PBS, resuspended in 420 μl of PBS, and then 980 μl of cold 100% ethanol was added dropwise into each tube while the tubes were being vortexed at slow speed. The ethanol-fixed cells were stored at –20°C until needed. Fixed cells were centrifuged between 7,000 rpm and 8,000 rpm for 5 minutes after which the pellet was resuspended in 500 μl of PBS/RNAse (final concentration, 0.1 mg/ml). The cells

were incubated at 37°C for 15 minutes, mixed with 500 µl of PBS containing propidium iodide at a final concentration of 25 µg/ml, and analyzed by FACScan cytofluorometer (Becton Dickinson, San Jose, CA)

Hoechst staining for apoptotic nuclei

HEK 293T cells were seeded in six-well plates at 1 x 10^5 cells per well the day before transfection, when cells were transfected with 2 µg DNA via liposomes and incubated at 37°C for 24 h. At that time, Hoechst 33342 (Sigma, St. Louis, MO, USA) was added into the medium to a final concentration of 0.25 µg/ml. The cells were incubated for 2 h at 37°C and then viewed by inverted fluorescence microscopy with the filter usually used to detect nuclei stained with 4',6-diamidino-2-phenylindole. Normal and apoptotic nuclei were counted from three randomly chosen fields, and the percentage of apoptotic cells was determined from those counts.

Cell viability assays

A luciferase-based *in vitro* cell viability assay was used. Briefly, HEK 293T and the breast cancer cell lines MCF-7, MDA-MB-435, and MDA-MB-231 were co-transfected with either wild-type hBok or NES-mutant hBok together with 10% fractions of the indicator plasmid CMV luciferase expressing vector, with liposomes used as the gene-delivery vehicle. Inclusion of a ten-fold excess of the hBok expression vector as compared with the pLuc reporter plasmid was done to ensure that most of the pLuc-expressing cells would also express hBok. At 24 h after transfection, cells were lysed and luciferase activity determined. Standard deviations were calculated from three independent experiments. Cell viability was also analyzed by transfecting the cells with the wild-type or mutant hBok cloned into the AdTrack vector so that both the hBok

construct and GFP could be expressed independently from the same expression vector. At 24 h after transfection, the cells were washed and the percentage of GFP-expressing cells from the population of cells in each field determined by microscopic analysis. The percentage of cell viability was normalized according to the number of green cells in the population transfected, with the vector-only condition used as the baseline (100%).

Western blot analysis

Proteins were extracted from the cells by using protein lysis buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 0.5% sodium deoxycholate and 1% NP40, containing protease inhibitors; 1μg/ml aprotinin, 0.5 mM PMSF, 0.1M NaF, 2mM Na_3VO_4 and 1 mM benzamidine. Proteins (50 μg per sample) in SDS-loading buffer (100 mM Tris [pH 6.8], 200 mM dithiothreitol, 4% SDS, 20% glycerol and 0.2% bromophenol blue) were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to Immun-Blot PVDF [polyvinylidene fluoride] Membranes (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 5% dry milk and 0.1% Tween 20 (U.S. Biochemical, Cleveland, OH, USA) in PBS followed by incubations with Anti-Flag M5 monoclonal antibody (Sigma, St. Louis, MO, USA)) and then with horseradish peroxidase-conjugated secondary antibody according to the manufacturer's instructions. The immunoblots were visualized by using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech).

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Abbreviations

BH	Bcl-2 homology
CMV	cytomegalovirus
FACS	fluorescence-activated cell sorting
GFP	green fluorescence protein
hBok	human Bcl-2–related ovarian killer
HEK	human epithelial kidney
NES	nuclear export signal
PBS	phosphate-buffered saline
rBok	rat Bcl-2–related ovarian killer
SDS	sodium dodecyl sulfate
TUNEL	terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling

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Res., **62,** 8-12.

LEGENDS TO FIGURES

Figure 1 Determining the apoptotic potential of hBok in HEK 293T cells. **(a)** Cells growing in two-chamber slides (1 x 10^5 cells/ chamber) were transiently transfected with pcDNA3 (vector) or pcDNA3 expressing rBok or hBok. Apoptotic cells (arrows) were identified by TUNEL assay. Results in the bar graph are shown as the median ± standard error from 3 independent experiments. **(b)** HEK 293T cells (1 x 10^6 cells / 100-mm plate) were transiently transfected with pcDNA3 (vector control) or pcDNA3 expressing rBok or hBok and apoptosis determined by DNA fragmentation analysis. **(c)** HEK 293T cells (1 x 10^6 cells /100-mm plate) were transiently transfected with pcDNA3 (vector control) or pcDNA3 expressing rBok or hBok, and 24 h later cells (in the medium) were treated with Hoechst 33342 for 2 h. Arrows indicate apoptotic cells. Percentages of apoptotic cells were calculated by counting both normal and apoptotic cells from three randomly chosen fields; results in the bar graph are shown as the median ± standard error from 3 independent experiments. **(d)** HEK 293T cells (1 x 10^6 cells / 100-mm plate) were transiently transfected with pcDNA3 (vector control) or pcDNA3 expressing hBok or rBok and apoptosis was determined by FACS analysis 24 h after transfection. TUNEL assay:- hBok induced apoptosis ~ 30% vs ~20% for rBok, Hoechst Staining:- hBok induced apoptosis~24% vs 16% for rBok, and FACS Analysis:- hBok induced apoptosis ~11% vs ~5% for rBok.

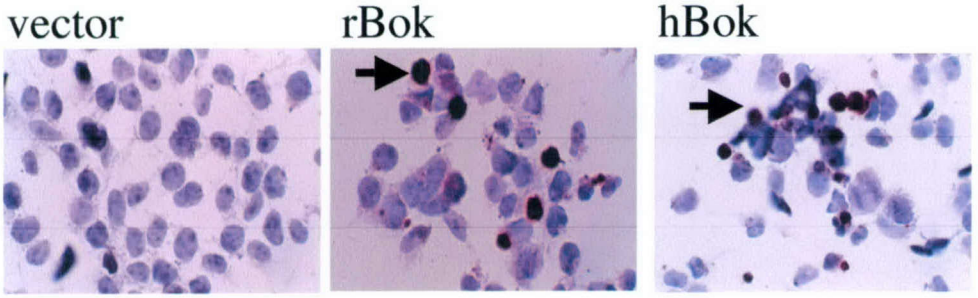
Figure 2 Nuclear translocation of hBok. (**a**) Western blot analysis of nuclear and cytoplasmic fractions of HeLa and MDA-MB-231 cells showing the localization of endogenous Bok and Bax.

(b,c) HeLa and MDA-MB-231 cells (3 x 10^5 cells / chamber on 4-chamber slides) were transiently transfected with Flag-tagged hBok (0.6 μg). Immunofluorescence staining reveals hBok in the nucleus (arrows) independent of its localization in the endoplasmic reticulum. **(d)** Western blot analysis of nuclear and cytoplasmic fractions of transfected HEK 293T cells with antibodies against Flag, PARP, and α-tubulin.

Figure 3 Nuclear export of hBok depends on Crm1. **(a)** Open reading frame of hBok highlighting the putative nuclear export sequence (LLRLGDELE) within the BH3 domain. **(b)** Crm1 was immunoprecipitated (IP) and associated hBok was determined by immunoblotting (IB) (left panel). Conversely, Bok was immunoprecipitated (IP) and associated Crm1 was determined by immunoblotting (IB) (right panel). **(c)** Leptomycin B sequesters hBok in the nucleus. MDA-MB 231 cells (3 x 10^5 cells / chamber in 4-chamber slides) were transiently transfected with Flag-tagged hBok (0.6 μg) and harvested 16 h later. One set of transfected cells (–) was left untreated and the other (+) was incubated with 20 ng/ml leptomycin B (LMB) for another 6 h. Quantitative analysis of the immunofluorescence staining shows an increase in nuclear hBok after treatment with leptomycin B. **(d)** The NES mutant was generated by substituting the amino acid residues ^{72}LRL^{74} with alanine. **(e)** Mutation of the putative NES of hBok sequesters the protein in the nucleus and enhances its killing potential. MDA-MB-231 cells (3 x 10^5 cells / chamber in 4-chamber slides) were transiently transfected with Flag-tagged NES mutant hBok (0.6 μg) and harvested 16 h after transfection. (Later time points resulted in apoptotic cells being washed off the slide during the staining procedure). Quantitative analysis of the immunofluorescence staining shows a strong staining of hBok in the nucleus of cells transfected with the NES mutant.

Figure 4 Accumulation of Bok in the nucleus induces a strong apoptotic response. **(a)** HEK 293T cells were transiently cotransfected with pcDNA3 (control, not shown) or pLuc plus phBok or NES mutant plus pLuc. Cell viability was determined with a luciferase reporter assay. **(b)** HEK293T cells ($1x10^6$ cells/100 mm plate) transiently transfected with pcDNA3 (vector) and vector expressing either wild-type hBok or NES mutant hBok were harvested at the times indicated and cell viability determined by trypan blue exclusion. **(c)** HEK 293T cells (1×10^6 cells / 100-mm plate) were transiently transfected with pcDNA3 (vector control) or pcDNA3 expressing either hBok or NES-mutant hBok. The percentage of apoptotic cells was determined by FACS analysis at 24 h after_transfection. The percentage of apoptotic cells is indicated in each set. **(d)** MDA-MB-435 breast cancer cells (1×10^6 cells / plate) were transfected with 10 μg AdTrack or vector expressing either wild-type or the NES-mutant hBok so that both Bok and GFP could be expressed from the same vector. Percentages of GFP-expressing cells were determined by fluorescence microscopy from three separate fields to determine the percentage of cell death and quantitatively analyzed by comparing the number of viable cells in the Bok-transfected cells to the number of viable cells transfected with the empty vector.

a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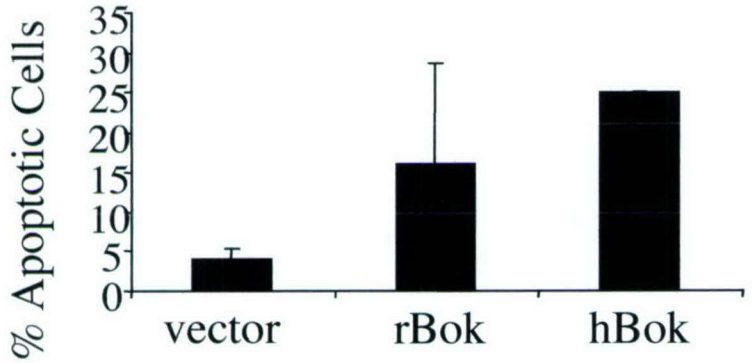
rBok
hBok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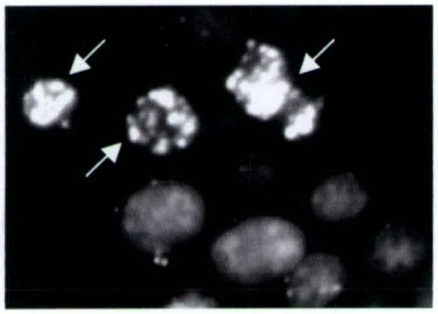

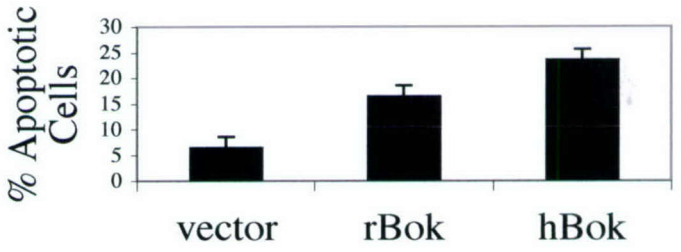
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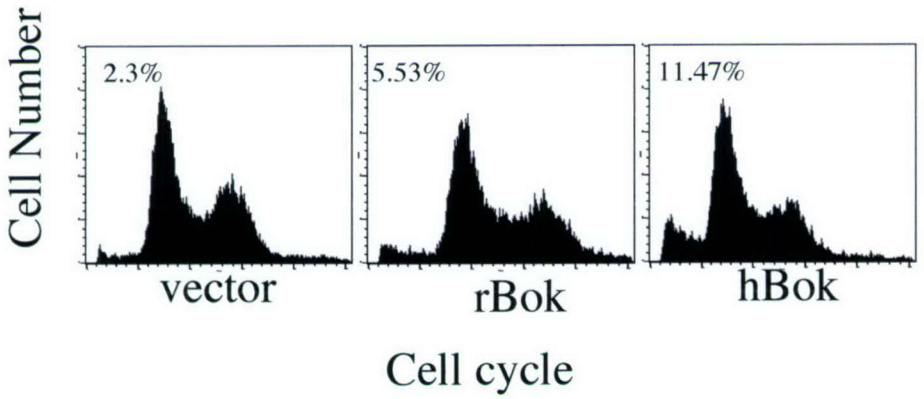
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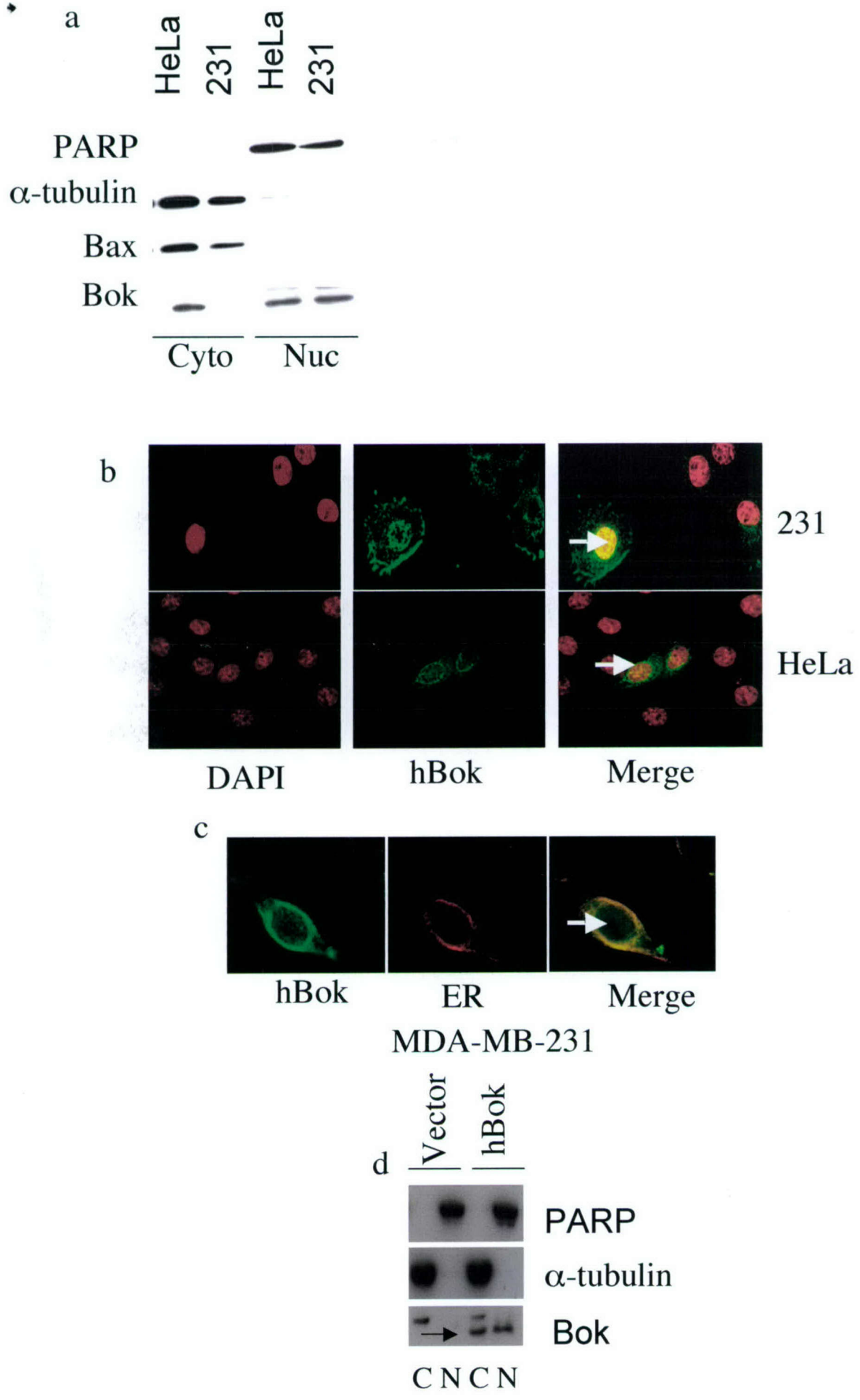
5.53%
11.47%
vector
rBok
hBok
Cell cycle

Fig. 1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hBok
ER
Merge
MDA-MB-231
d
Vector
hBok
PARP
α-tubulin
Bok
C N C N

Fig. 2

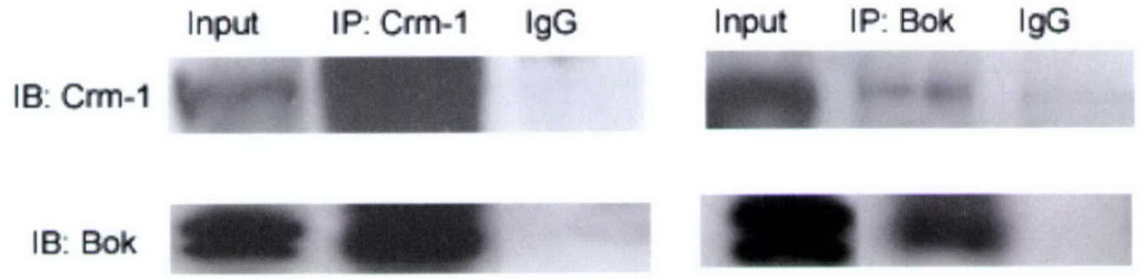
a

Typical NES

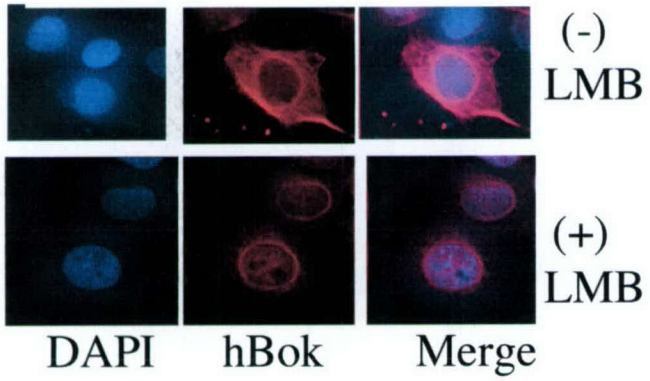
HIV-1 Rev	**LQLPPLERLTL**	Bok*	^{71}**LLRLGDELE**^{79}
PKI	**LALKLAGLDIG**	*Sequence is identical in human, rat,	
p53	**MFRELNEALELK**	mouse, and chicken Bok	

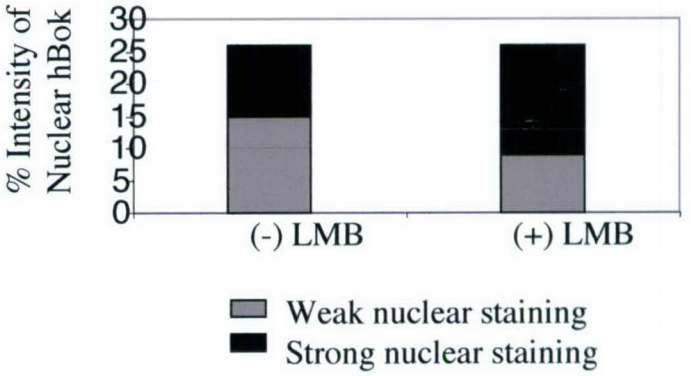
Consensus sequence
L-X2-3-L-X2-3-L-X-L

b



c

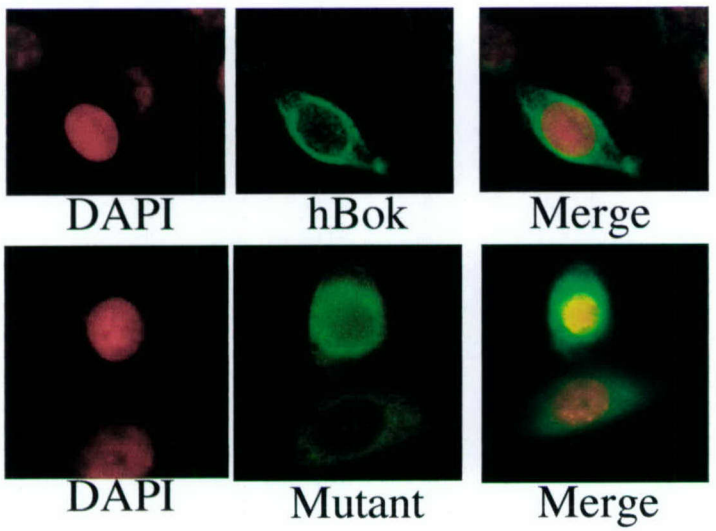




d

Bok	^{71}**LLRLGDELE**^{79}
Mutant	^{71}**LAAAGDELE**^{79}

e



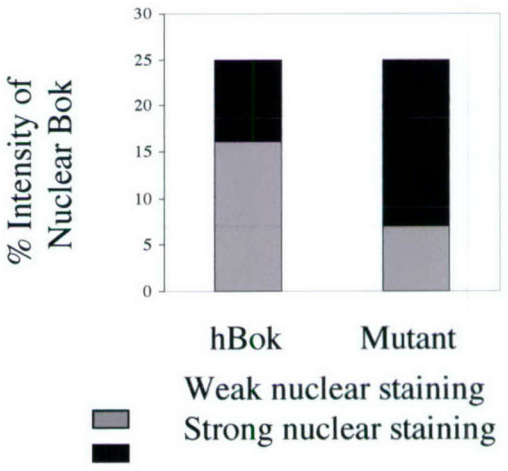


Fig. 3

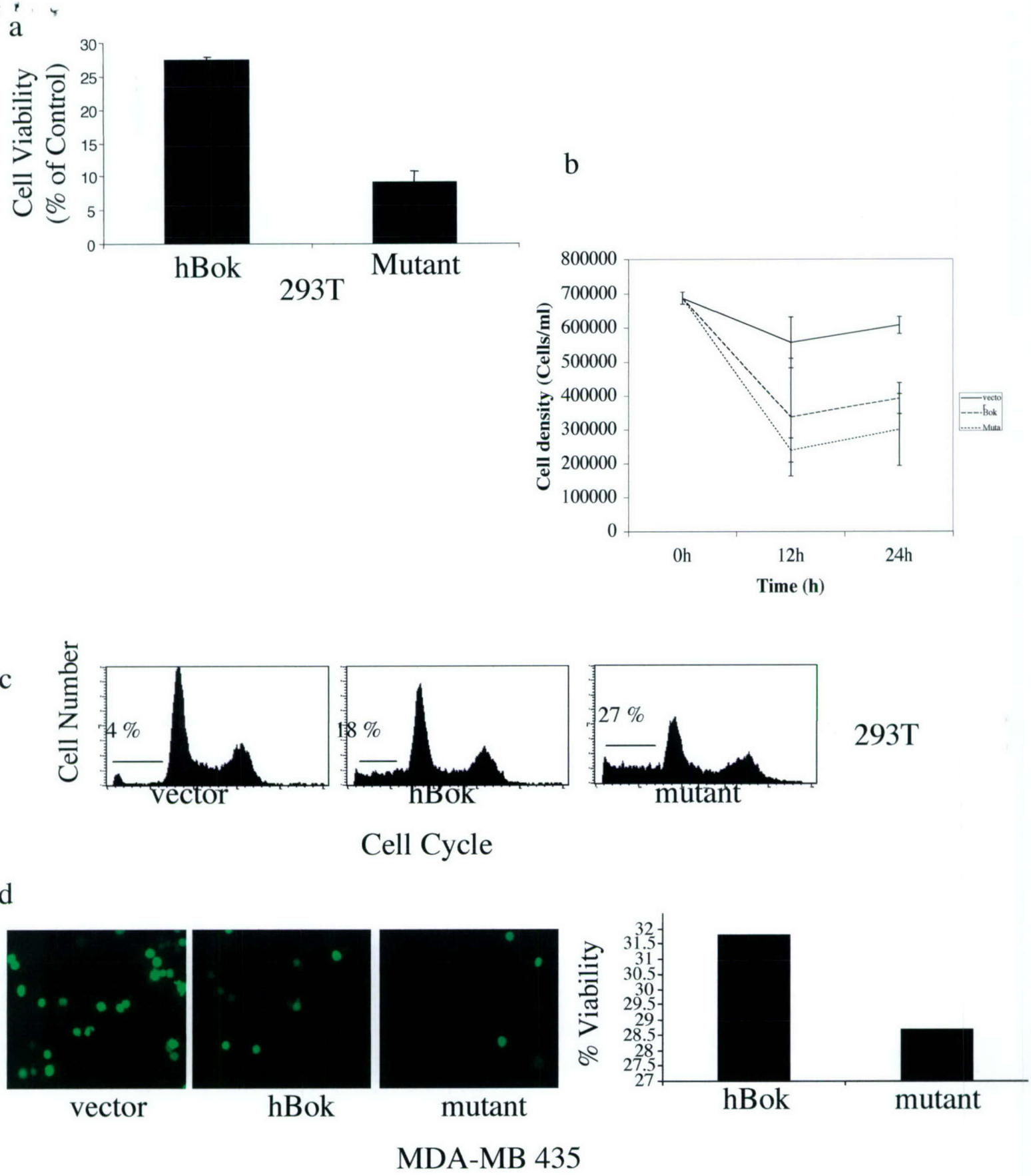

a
Cell Viability (% of Control)
30
25
20
15
10
5
0
hBok
Mutant
293T
b
Cell density (Cells/ml)
800000
700000
600000
500000
400000
300000
200000
100000
0
0h
12h
24h
Time (h)
vecto
Bok
Muta
c
Cell Number
4 %
18 %
27 %
vector
hBok
mutant
293T
Cell Cycle
d
vector
hBok
mutant
% Viability
32
31.5
31
30.5
30
29.5
29
28.5
28
27.5
27
hBok
mutant
MDA-MB 435

Fig. 4